

Development of an electrochemical immunosensor for the detection of HbA1c in serum†‡

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Received 31st October 2011, Accepted 19th December 2011

DOI: 10.1039/c2an16034j

An electrochemical immuno-biosensor for detecting glycosylated haemoglobin (HbA1c) is reported based on glassy carbon (GC) electrodes with a mixed layer of an oligo(phenylethynylene) molecular wire (MW) and an oligo(ethylene glycol) (OEG). The mixed layer is formed from *in situ*-generated aryl diazonium cations. To the distal end of the MW, a redox probe 1,1'-di(aminomethyl)ferrocene (FDMA) was attached followed by the covalent attachment of an epitope *N*-glycosylated pentapeptide (GPP), an analogon to HbA1c, to which an anti-HbA1c monoclonal antibody IgG can selectively bind. HbA1c was detected by a competitive inhibition assay based on the competition for binding to anti-HbA1c IgG antibodies between the analyte in solution, HbA1c, and the surface bound epitope GPP. Exposure of the GPP modified sensing interface to the mixture of anti-HbA1c IgG antibody and HbA1c results in the attenuation of ferrocene electrochemistry due to free antibody binding to the interface. Higher concentrations of analyte led to higher Faradaic currents as less anti-HbA1c IgG is available to bind to the electrode surface. It was observed that there is a good linear relationship between the relative Faradaic current of FDMA and the concentration of HbA1c from 4.5% to 15.1% of total haemoglobin in serum without the need for washing or rinsing steps.

Introduction

HbA1c is a stable minor haemoglobin variant formed by a non-enzymatic reaction of glucose with N-terminal valine of an adult haemoglobin's β chain in the human body. Thus, HbA1c is the main diabetes marker protein for monitoring a person's average blood sugar level over the preceding 2 to 3 months.¹ Based on a direct relationship between HbA1c and diabetic complications, recent guidelines for the management of diabetes now stress the importance of HbA1c monitoring.^{2,3} The non-enzymatic origin makes the direct analysis of glycosylated haemoglobin more difficult in comparison with the diagnosis of other analytes that involve enzymatic reactions. Due to the importance of HbA1c monitoring, numerous analytical methods were developed for its analysis, such as cation-exchange,^{4,5} boronate affinity chromatography,^{6–8} agarose gel electrophoresis,⁹ colorimetric methods,^{10,11} spectroscopic measurements,^{12–14} and mass

spectroscopy.¹⁵ For clinical analyses, boronate-affinity chromatography and cation-exchange chromatography in particular are widely used.¹⁶ High performance liquid chromatography (HPLC), is still considered the standard reference method however. Nevertheless, the main issue with these methods are they tend to be time-consuming, expensive, and can give false results due to the coexistence of genetic variants and other chemically modified derivatives of haemoglobin.²⁵

Electrochemical affinity sensors have considerable potential with regards to developing portable analytical devices for detecting HbA1c. This is because electrochemical methods are compatible with miniaturization and with measurements in complex samples such as blood.¹⁷ However, the majority of these sensing interfaces are based on boronate-affinity^{7,18–23} and involve enzyme labelling.^{8,24} To date the more reliable method of distinguishing the different variants of haemoglobin is using antibodies that are specific for the glycosylated amino terminus of the β chain or other adducts.^{26,27} Hence the development of an electrochemical immunosensors for detecting HbA1c with high selectivity and simplicity is required.

In this study, a competitive inhibition assay for detecting HbA1c was developed using a *N*-glycosylated pentapeptide (GPP) as an HbA1c analogon. The proposed immunosensor is inspired by a recently developed electrochemical immunosensor based on the modulation of amperometric signals of surface bound redox species when immersed in a protein environment.²⁸ With this immunosensor a mixed layer of molecular wire (MW)

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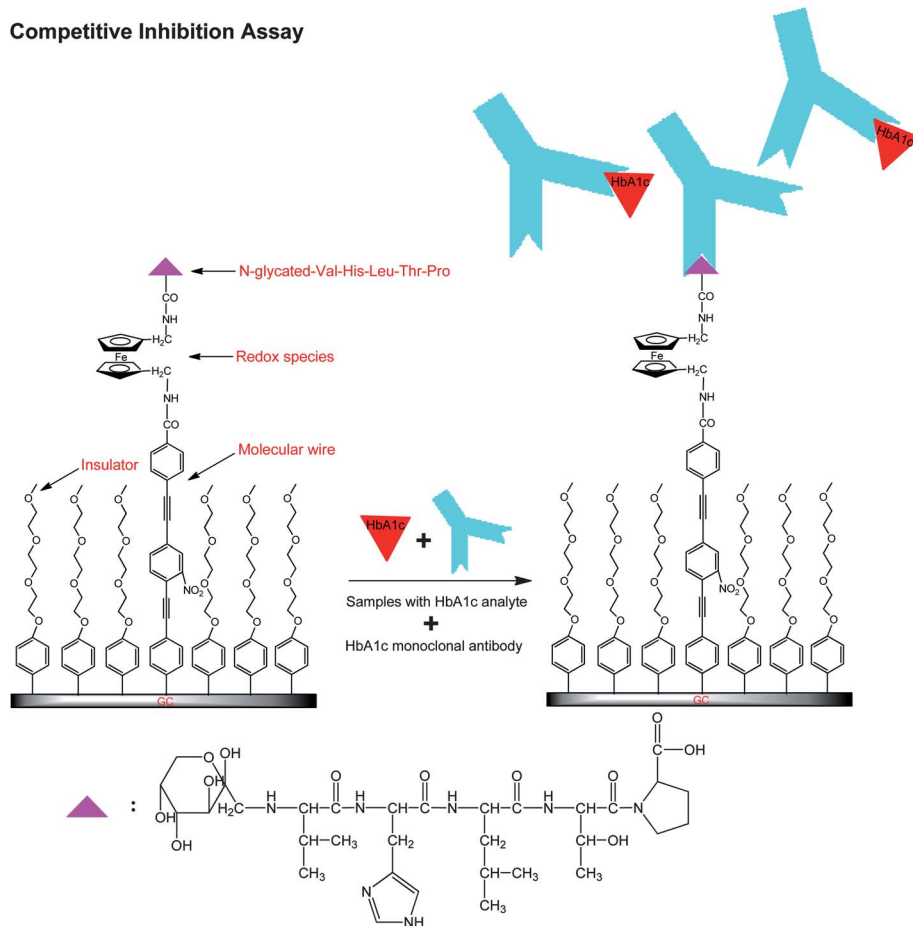
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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2an16034j

and oligo(ethylene glycol) (OEG) was attached onto a glassy carbon (GC) electrode. 1,1'-di(aminomethyl)ferrocene (FDMA) was then attached to the end of the MW followed by an epitope (the structural feature an antibody selectively recognises) to which an antibody binds to give the final fabricated immunosensor interface. In the work presented here, the epitope employed is a pentapeptide, glycolysated-VHLTP (GPP). Transduction in this system is based on the amperometric signal of surface bound ferrocene moiety being attenuated when antibodies bind to the epitope due to the immersion of the ferrocene into a protein environment. This immunosensing concept is applicable to either the detection of antibodies²⁸ or the detection of small molecules^{28–30} such as drugs and pesticides. Here the concept is applied to the detection of HbA1c. Unlike small molecules that the system was designed to detect, HbA1c and haemoglobin actually represent the detection of large proteins. Hence, this study represents a broadening of the scope of the thus far reported immunosensing technology. In this study, a competitive inhibition assay for the detection of HbA1c has been investigated as shown in Scheme 1.

The procedure for fabricating the sensing interface (Scheme 1) is described in the supporting information.[†] Briefly, after the surface modification of a GC electrode with mixed layer of MW/

OEG, where the two components were mixed in solution with a molar ratio of 1 : 50, FDMA was attached to the free carboxylic acid moiety at the end of the MW. Characterization of this interface, including the amount of FDMA and other species attached, was performed previously.³⁰ To the distal free amine of FDMA, the GPP was attached using the classical carbodiimide coupling. Note that the GPP (Scheme 1) contains no free amine and only a single carboxyl group. Therefore only one coupling reaction can occur and a well-defined sensing interface is achieved. After the attachment of GPP, the electrochemistry of FDMA modified GC electrode surfaces showed only a minor attenuation in current intensity. This result is encouraging as it indicates the peptide does not block the surface electrochemistry, a necessary condition for the sensor to be able to operate. Complexation of the human anti-HbA1c monoclonal IgG antibody with the GPP attached to the distal end of the MW results in an obvious attenuation of the ferrocene electrochemistry (Fig. 1), as observed previously for other antibody-epitope combinations.^{28,29} In this study, the anti-HbA1c IgG antibody binding to the sensing interface results in the FDMA electrochemistry being suppressed by $67\% \pm 4\%$ (95% confidence, $n = 6$) of the original value prior to exposure to the anti-HbA1c IgG antibody.



Scheme 1 Schematic of the design for a competitive inhibition assay for detecting HbA1c. The anti-HbA1c IgG antibody is represented by the blue Y, the red triangle represents HbA1c and the pink surface bound triangle is the glycolysated pentapeptide. The structure of GPP (*N*-glycosylated-VHLTP) is inserted.

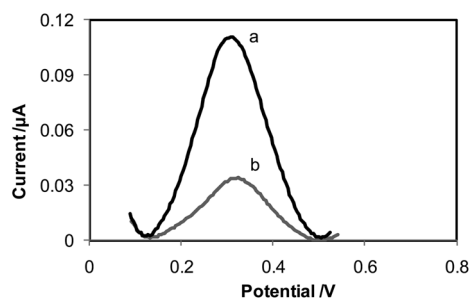


Fig. 1 Representative square wave voltammograms (SWV) for FDMA modified GC electrodes after the stepwise attachment of (a) GPP, and (b) anti-HbA1c IgG antibody.

Three controls were performed to verify that the change in current was due to a specific interaction between the anti-HbA1c antibody IgG and the surface bound GPP epitope. These were 1) the carboxyl on the GPP was not activated with EDC/NHS and hence did not couple to the MW. 2) the anti-HbA1c antibody IgG was pre-complexed with 2 mM GPP for 3 h at 4 °C, such that the antibody had no available binding sites to complex with the surface and 3) the biosensing interface was incubated in either the wrong IgG, in this case 10 $\mu\text{g mL}^{-1}$ anti-biotin IgG antibody, or different type of proteins, such as 10 $\mu\text{g mL}^{-1}$ of bovine serum albumin. In all three controls only a minor decrease in current (5% of current intensity determined by SWV) was observed.

As a consequence of the anti-HbA1c IgG antibody selectively binding to the sensing interface, a competitive inhibition assay (Scheme 1) was developed for detecting the amount of HbA1c in human serum. In a competitive inhibition assay, the final biosensing interface is the FDMA and GPP attached to the MW in the absence of anti-HbA1c IgG antibody. The anti-HbA1c IgG antibody is then introduced into a sample solution where it can complex with the analyte present. Any remaining uncomplexed anti-HbA1c IgG is then free to bind with the epitope and the FDMA electrochemistry was attenuated. Hence the greater the amount of analyte, the more anti-HbA1c IgG complexes with the analyte, the lower the amount of free anti-HbA1c IgG available to bind with the epitope and thus the higher the electrochemical signal. In order to verify that the competitive inhibition assay system is viable to use in a complex matrix such as human serum, the sensing interface was incubated in the serum containing 2 $\mu\text{g mL}^{-1}$ of anti-HbA1c IgG antibody and 13.5% of

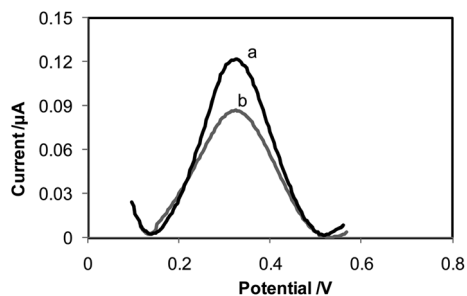


Fig. 2 A representative SWV for FDMA modified GC electrodes after (a) the attachment of GPP, and (b) the subsequent incubation in a mixture containing 2 $\mu\text{g mL}^{-1}$ of anti-HbA1c IgG antibody and 13.5% HbA1c.

HbA1c (Fig. 2). As can be seen from Fig. 2 there is some current attenuation, suggesting there is free antibody in solution that has not complexed with the HbA1c, and hence can bind to the sensing interface. The current decreased by $29\% \pm 2\%$ (95% confidence, $n = 6$). This is a significantly lower current attenuation (29%, Fig. 2) compared with that observed when the GPP modified interface was exposed to anti-HbA1c IgG (67%, Fig. 1) in the absence of the analyte (HbA1c). The lower current attenuation in the presence of the HbA1c indicates that some of the anti-HbA1c IgG antibody is complexed with the HbA1c and hence cannot bind to the sensing interface.

HbA1c standards, with percentages of HbA1c of 4.5%, 8%, 12.1% and 15.1%, but with the same total amount of haemoglobin (glycosylated and non-glycosylated) were used to construct a calibration curve. Note that these HbA1c standards were prepared in human serum. HbA1c samples with other concentrations were also prepared by mixing the stock solution of HbA1c standard R1 (4.5%) and another stock solution of HbA1c standard, R4 (15.1%) in different molar ratios. The calibration curve was plotted as shown in Fig. 3 and the studied HbA1c concentration range has covered the expected clinical range of haemoglobin. Fig. 3 shows that the relative current obtained is linear within the range of 4.5% to 15.1% (the percentage of HbA1c to the total amount of haemoglobin).

In control experiments, where the sensing interface was exposed to a mixture of anti-HbA1c antibody IgG and 2 mM of pentapeptide VHLTP (peptide that was not glycosylated), or was exposed to haemoglobin, the current attenuation was comparable to when the sensor was incubated in a serum only containing the anti-HbA1c IgG and no HbA1c (See Fig. S4 for example).

In summary, an electrochemical immunosensor has been developed for detecting HbA1c. Firstly, GC electrodes were modified with mixed layer of MW and OEG. Then the redox species FDMA was covalently attached to the interface followed by the attachment of an epitope, a glycosylated pentapeptide. The binding of anti-HbA1c IgG to this sensing interface causes the attenuation of the FDMA electrochemistry. A competitive inhibition assay was employed for detecting HbA1c within the clinical range (5%–20%) in undiluted human serum. A linear relationship was obtained between the relative current and the

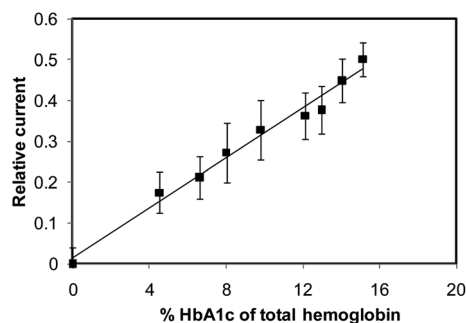


Fig. 3 Calibration curve for HbA1c measured with the competitive inhibition assay. The total concentration of haemoglobin was about 15 g dL^{-1} . Four calibrators were pre-mixed with 2 $\mu\text{g mL}^{-1}$ of anti-HbA1c monoclonal antibody IgG for 30 min at 4 °C, respectively. The mixture was then drop-coated onto the GPP modified sensing interface. Each data point represents the average value of six GC electrodes.

concentration of HbA1c. The sensing system provides a sensitive and applicable approach for detecting HbA1c.

This work was supported financially by the Australia Research Council Linkage Grant scheme (LP0775216 and LP110200610).

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